Insulin-stimulated protein kinase B phosphorylation on Ser-473 is independent of its activity and occurs through a staurosporine-insensitive kinase

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Abstract: Full activation of protein kinase B (PKB, also called Akt) requires phosphorylation on two regulatory sites, Thr-308 in the activation loop and Ser-473 in the hydrophobic C-terminal regulatory domain (numbering for PKB alpha/Akt-1). Although 3'-phosphoinositide-dependent protein kinase 1 (PDK1) has now been identified as the Thr-308 kinase, the mechanism of the Ser-473 phosphorylation remains controversial. As a step to further characterize the Ser-473 kinase, we examined the effects of a range of protein kinase inhibitors on the activation and phosphorylation of PKB. We found that staurosporine, a broad-specificity kinase inhibitor and inducer of cell apoptosis, attenuated PKB activation exclusively through the inhibition of Thr-308 phosphorylation, with Ser-473 phosphorylation unaffected. The increase in Thr-308 phosphorylation because of overexpression of PDK1 was also inhibited by staurosporine. We further show that staurosporine (CGP 39360) potently inhibited PDK1 activity in vitro with an IC(50) of approximately 0.22 microm. These data indicate that agonist-induced phosphorylation of Ser-473 of PKB is independent of PDK1 or PKB activity and occurs through a distinct Ser-473 kinase that is not inhibited by staurosporine. Moreover, our results suggest that inhibition of PKB signaling is involved in the proapoptotic action of staurosporine.

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Insulin-stimulated protein kinase B phosphorylation on Ser473 is independent of its activity and occurs through a staurosporine-insensitive kinase

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Running Title: Ser473 phosphorylation by a staurosporine-insensitive kinase

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Summary

Full activation of protein kinase B (PKB, also called Akt) requires phosphorylation on two regulatory sites: Thr308 in the activation loop and Ser473 in the hydrophobic C-terminal regulatory domain (numbering for PKBα/Akt-1). While 3-phosphoinositide-dependent protein kinase 1 (PDK1) has now been identified as the Thr308 kinase, the mechanism of the Ser473 phosphorylation remains controversial. As a step to further characterize the Ser473 kinase, we examined the effects of a range of protein kinase inhibitors on the activation and phosphorylation of PKB. We found that staurosporine, a broad-specificity kinase inhibitor and inducer of cell apoptosis, attenuated PKB activation exclusively through the inhibition of Thr308 phosphorylation, with Ser473 phosphorylation unaffected. The increase in Thr308 phosphorylation due to over-expression of PDK1 was also inhibited by staurosporine. We further show that staurosporine (CGP 39360) potently inhibited PDK1 activity *in vitro* with an IC_{50} of ~0.22 μM. These data indicate that agonist-induced phosphorylation of Ser473 of PKB is independent of PDK1 or PKB activity, and occurs through a distinct Ser473 kinase which is not inhibited by staurosporine. Moreover, our results suggest that inhibition of PKB signaling is involved in the pro-apoptotic action of staurosporine.

Introduction

Protein kinase B (PKB) is activated by growth factors in a phosphoinositide 3-kinase (PI3K)-dependent manner, through translocation to the plasma membrane and phosphorylation on two regulatory sites, Thr308 in the activation loop in the kinase domain, and Ser473 in the hydrophobic C-terminal regulatory domain (1). Phosphorylation on both sites are required for full activation of PKB, however, the contribution of each site towards PKB activation is not equal. Thus, while phosphorylation on Thr308 alone is able to increase PKB activity, phosphorylation on Ser473 alone does not significantly stimulate the kinase (1,2). While the upstream kinase responsible for phosphorylation of Thr308 has been identified as 3'-phosphoinositide-dependent kinase-1 (PDK1), the identity of the Ser473 kinase has yet to be determined (3,4).

Mitogen-activated protein kinase-activated protein kinase-2 (MAPKAPK-2) was the first kinase shown to phosphorylate PKBα on Ser473 *in vitro* (1). However, MAPKAPK-2 is unlikely to be the physiological Ser473 kinase as it is activated downstream of p38 mitogen-activated kinase (p38 MAPK) in response to stress, in a PI3K-independent manner, and inhibition of p38 MAPK by SB 203580 did not interfere with activation of PKB (1). Integrin-linked kinase (ILK) was also shown to phosphorylate Ser473 on PKBα *in vitro*, and overexpression of a kinase-inactive ILK inhibited Ser473 phosphorylation (5). However, certain kinase-inactive ILK mutants also induced Ser473 phosphorylation, suggesting that ILK is unlikely to be the direct Ser473 kinase *in vivo* (6). Two further candidate Ser473 kinases have been recently proposed: PDK1 (7) and PKB itself (8). PDK1, in the presence of a peptide

resembling the phosphorylated Ser473 region of PKB is able to phosphorylate Ser473, in addition to Thr308 of PKBα (7). However, PDK1 is clearly not the *in vivo* Ser473 kinase, as PDK1-null embryonic stem cells are impaired in Thr308, but not Ser473 phosphorylation (9). Autophosphorylation was originally ruled out because kinase-inactive PKBα undergoes insulin-like growth factor-1 (IGF-1)-induced phosphorylation at both Thr308 and Ser473 when overexpressed in human embryonic kidney (HEK) 293 cells (1). In contrast to these observations, Toker and Newton recently demonstrated that IGF-1 stimulated phosphorylation of kinase-inactive PKBα on Thr308 but not on Ser473 when overexpressed in the same cells, and that PKBα is able to autophosphorylate on Ser473 *in vitro* (8). Thus, it seems possible that agonist-induced Ser473 phosphorylation may be mediated by PKB itself.

To further characterize the upstream kinase(s) involved in the activation of PKB, we have adopted a pharmacological approach by screening for protein kinase inhibitors which differentially inhibit either Thr308 or Ser473 phosphorylation. We found that staurosporine, a broad-specificity protein kinase inhibitor, attenuated PKB activation specifically through inhibition of PDK1, with an IC_{50} of ~0.22 μM *in vitro*. Staurosporine has been widely used as an inducer of apoptosis, however, the cellular target(s) of its pro-apoptotic action are not known. Our data suggest that at least part of the apoptotic effects of staurosporine is due to inhibition of PKB signaling. In contrast to Thr308 phosphorylation, insulin-stimulated phosphorylation of the Ser473 site was not reduced by staurosporine treatment (up to 1 μM). Taken together, our results suggest that phosphorylation of PKB on Ser473 does not occur by autophosphorylation, but rather through the action of an upstream kinase which is

resistant to staurosporine and distinct from PDK1.

Experimental Procedures

Expression constructs and transfection of cells

Culture and transfection of HEK 293 cells, and the expression constructs used in this study have been previously described (1,10-12).

Recombinant proteins

Expression and infection of insect Sf9 cells have been described previously for PKB and PKC (13,14). Human PDK1-glutathione S-transferase fusion protein (GST-PDK1) was cloned in a modified pFastBac vector (Life Technologies), and prepared as previously described (13).

Immunoprecipitation, immunoblotting and in vitro kinase assays

Cell lysis, immunoprecipitation, immunoblotting and PKB assay using crosstide peptide (GRPRTSSAEG) were performed as previously described (11). Phospho-specific PKB antibodies were purchased from Cell Signaling Technologies. In addition, we also produced and purified an anti-phospho-Ser473 PKB antibody using the peptide Arg-Pro-His-Phe-Pro-Gln-Phe-Ser(PO_3H_2)-Tyr-Ser-Ala-Ser (15).

Assays for recombinant PKCα and PKCζ have been described previously (14). Recombinant GST-PDK1 was similarly assayed, using 0.1 mg/ml casein (Sigma) as substrate.

Results

We have previously reported the characterization of a PKBα construct in which the pleckstrin homology (PH) domain was replaced by the C1 domain of PKC (C1-PKBα-ΔPH) (11). C1-PKBα-ΔPH translocated to the membrane upon stimulation with the phorbol ester 12-*O*-tetradecanoylphorbol 13-acetate (TPA), and was activated and phosphorylated on both Thr308 and Ser473 (11). TPA-stimulated activation of C1-PKBα-ΔPH was inhibited by PI3K inhibitors, as well as the broad range protein kinase inhibitor, staurosporine (11). Interestingly, while the former inhibited phosphorylation on both sites, staurosporine treatment specifically attenuated phosphorylation on Thr308 without affecting Ser473 phosphorylation (11).

To extend our observations, the effect of a panel of protein kinase inhibitors was compared with staurosporine (Fig. 1). Staurosporine obtained from Alexis or Novartis (CGP 39360) inhibited TPA-stimulated Thr308 phosphorylation and activation of C1-PKBα-ΔPH, without affecting Ser473 phosphorylation (Fig. 1B). CGP 39360 was more potent than staurosporine (Alexis), requiring 0.1 and 1 μM to reduce kinase activity and Thr308 phosphorylation to basal levels, respectively (Fig. 1B). This difference may be due to improved purity of the chemical produced by Novartis. The staurosporine-derivative CGP 41251 also inhibited Thr308 phosphorylation and activation of C1-PKBα-ΔPH, but was much less potent than CGP 39360 (Fig. 1B). The inactive analog to CGP 41251, CGP 42700 had no significant effect (Fig. 1B). Three other protein kinase inhibitor compounds examined (CGP 25956, CGP 45910 and CGP 57148B) had an inhibitory effect only at concentrations above 10 μM, where they reduced phosphorylation at both sites to

below basal levels (Fig. 1C). Notably, an effect of CGP 57148B (STI571 or Glivec), was only observed at 40 μM (Fig. 1C). CGP 57148B is a potent inhibitor of Abl and PDGF receptor tyrosine kinases which selectively inhibits the growth of Bcr/Abl-transformed cells (16), and is now in clinical trials for treatment of chronic myeloid leukemia. The effects of CGP 57148B were observed concentrations <10 μM, and our results show that it does not significantly affect the PDK1/PKB pathway at this concentration (Fig. 1C).

To extend our observations to wild type PKB, we examined the effect of staurosporine on insulin-stimulated activation of HA-PKBα expressed in HEK293 cells. Staurosporine treatment inhibited insulin-stimulated HA-PKBα activation in a dose-dependent manner, with complete inhibition observed at 1 μM (Fig. 2A). Similar to C1-PKBα-ΔPH, this inhibitory effect of staurosporine on HA-PKBα activity correlated with an inhibition of Thr308 phosphorylation (Fig. 2A). In contrast, phosphorylation on Ser473 was slightly enhanced with increasing concentrations of staurosporine (Fig. 2A). A similar inhibitory effect of staurosporine was observed for insulin-stimulated Thr308 phosphorylation of endogenous PKB in HEK293 cells (Fig. 2B). Two other modes of PKB activation were also examined: co-expression of PDK1 and constitutive membrane targeting. In agreement with previous results (3), co-expression of PDK1 with HA-PKBα resulted in a 3-fold increase in basal PKBα activity, together with constitutive phosphorylation of Thr308 (Fig. 2C). Treatment with staurosporine reduced Thr308 phosphorylation and kinase activity (Fig. 2C). Interestingly, over-expression of PDK1 also induced an increase in Ser473 phosphorylation, reaching ~10% of the insulin-stimulated levels, which was reduced

with staurosporine treatment, suggesting that it occurs through a mechanism different from insulin-stimulated Ser473 phosphorylation (Fig. 2C). Targeting of PKB to the plasma membrane using the Lck myristoylation/palmitylation signal (m/p-PKBα) results in the constitutive activation and phosphorylation of PKB (10). In contrast to Thr308 phosphorylation induced by insulin or co-expression of PDK1, staurosporine did not reduce Thr308 phosphorylation of m/p-PKBα (Fig. 2D). This observation indicates that dephosphorylation of PKB does not occur readily at the plasma membrane, and that the phosphorylation step is the target of staurosporine.

Staurosporine is a competitive inhibitor which is thought to bind in the ATP pocket of target protein kinases (17). The observed effect of on Thr308 may occur via direct inhibition of PDK1, or staurosporine could bind to PKB, thus blocking the access to the phosphorylation site in the catalytic domain. To distinguish between these possibilities, we determined the inhibitory profiles of the CGP inhibitor compounds using recombinant GST-PDK1, GST-PKBα, PKCα and PKCζ. CGP39360 (staurosporine) was most potent against PKCα (IC_{50} < 3 nM), but also inhibited PDK1 (IC_{50} = 0.22±0.09 μM), PKBα (IC_{50} = 0.83±0.19 μM) and PKCζ (IC_{50} = 1.03±0.37 μM) at higher concentrations. CGP 41251 selectively inhibited PKCα (IC_{50} = 0.04±0.02 μM), and also inhibited PDK1 (IC_{50} = 1.72±0.21 μM), but did not have significant effects on PKBα or PKCζ (up to 10 μM). These data suggest that the target of staurosporine and its derivative CGP 41251 is PDK1 rather than PKB. CGP 42700, CGP 25956, CGP45910 and CGP 57148B did not inhibit any of the four kinases tested.

To further examine the regulation of PKB activation by upstream kinases, the effect of staurosporine on insulin-stimulated phosphorylation and activity of kinase-inactive (K179A) or phosphorylation site mutants (T308A and S473A) of PKBα was examined (Fig. 3). In agreement with previously results (1), Thr308 and Ser473 phosphorylation occurred independently of each other upon insulin stimulation, as observed in the phosphorylation site mutants (Fig. 3). In addition, the kinase-inactive mutant (K179A) was phosphorylation on both Thr308 and Ser473 upon insulin stimulation (Fig. 3). Staurosporine treatment inhibited insulin-stimulated Thr308 phosphorylation on wild type, K179A and S473A PKBα (Fig. 3), but its effect on Ser473 phosphorylation of the different PKBα constructs was somewhat varied. Staurosporine at 0.1 and 1 μM did not inhibit insulin-stimulated Ser473 phosphorylation of wild type and T308A-PKBα, but even enhanced their phosphorylation, which was more readily observed in the T308A mutant (Fig. 3). Interestingly, insulin-stimulated Ser473 phosphorylation of kinase-inactive PKBα was inhibited by staurosporine at 1 μM, but not at 0.1 μM (Fig. 3). As staurosporine is a broad-specificity kinase inhibitor, it is possible that complex effects are observed due to inhibition of numerous kinases/pathways at higher doses.

Discussion

Phosphorylation at Thr308 and Ser473 is required for full activation of PKBα. While the Thr308 kinase has been identified (PDK1), the Ser473 kinase remains elusive. Most recently, it has been suggested that autophosphorylation may be the mechanism by which PKB is phosphorylated on Ser473, and that the previously reported phosphorylation of kinase-deficient PKB at this site is due to the activity of endogenous PKB (8). Our previous results with m/p PKBα (10) and C1-PKBα-ΔPH (11) suggest that both upstream kinases are present in a constitutively active form at the plasma membrane. As PKB is present largely in the cytosol prior to stimulation, it seems unlikely that PKB itself is the physiological Ser473 kinase. More directly, here we show that pretreatment with 1 μM staurosporine abolished insulin-stimulated PKB activation of both transiently expressed and endogenous PKB, without affecting Ser473 phosphorylation (Fig. 2). In addition, using phospho-specific antibodies, we have confirmed our previous results (1) that kinase-inactive PKBα can be phosphorylated on both Thr308 and Ser473 in response to insulin (Fig. 3). Taken together, these data do not support the hypothesis that phosphorylation on Ser473 occurs via autophosphorylation or trans-phosphorylation. Rather, it confirms the existence of a distinct Ser473 kinase which is constitutively active at the plasma membrane of quiescent cells.

One kinase that fulfills the above criteria is PDK1. Indeed, PDK1 has the ability to phosphorylate PKBα on Ser473 in the presence of an exogenous peptide which resembles phosphorylated Ser473 (7). However, PDK1 is not the physiological Ser473 kinase because PDK1-null embryonic stem cells are not impaired in Ser473

phosphorylation in response to IGF-1 (9), and staurosporine inhibits PDK1 activity without affecting insulin-stimulated Ser473 phosphorylation (Fig. 2). Interestingly, overexpression of PDK1 in HEK293 cells not only induced constitutive phosphorylation of PKB at Thr308, but also caused a slight elevation of Ser473 phosphorylation (Fig. 2C). In this case, however, Ser473 phosphorylation is dependent on PDK1 activity, as it is attenuated by staurosporine treatment (Fig. 2C), and overexpression of a kinase-inactive PDK1 mutant did not increase basal Ser473 phosphorylation in HEK293 cells (data not shown). Thus, while PDK1 is not the physiological Ser473 kinase, it likely plays a role in Ser473 phosphorylation, with the nature of this interaction yet to be defined.

Integrin-linked kinase (ILK) has recently come to attention as a prime candidate kinase for Ser473 phosphorylation (5). According to our observations, the Ser473 kinase should be staurosporine-resistant. Unfortunately, we were unable to determine the effect of staurosporine on ILK as we have not been able to detect any significant kinase activity of overexpressed or endogenous ILK by autophosphorylation, or on myelin basic protein (data not shown). Intriguingly, ILK possesses a hydrophobic motif similar to the Ser473 site, and when this serine was mutated to an acidic residue to mimic phosphorylation, the ability of a kinase-inactive ILK to induce Ser473 phosphorylation was rescued (6). It was recently shown that the hydrophobic phosphorylation site in p90 ribosomal S6 kinase-2 (RSK2) acts as a docking site for the recruitment of PDK1 (18). Thus, it is possible that ILK mediates the co-localization of PKB with PDK1 and the Ser473 kinase.

Staurosporine exhibits anti-proliferative properties on a wide range of mammalian cells, and its derivatives UCN-01, CGP 41251, Ro 31-8220 and PKC412 (19-22) are being examined as potential therapeutic agents for the treatment of cancer. Despite the common use of staurosporine as an inducer of apoptosis, the direct cellular target of staurosporine is not known. Our finding that staurosporine inhibits PDK1 activity raises the possibility that staurosporine and its derivatives may induce apoptosis by interfering with survival signaling mediated by PDK1. Indeed, it was recently shown that reduction of PDK1 expression by anti-sense oligonucleotides induced apoptosis (23). Apart from PKB, PDK1 also phosphorylates and activates other kinases that are involved in cell survival, including $p70^{S6K}$ (12), $p90^{RSK}$ (24) and SGK (25). Thus the mechanisms of staurosporine-induced apoptosis needs to be re-addressed in light of its effects on PDK1 activity.

In summary, we have demonstrated that staurosporine attenuates PKB activation through direct inhibition of PDK1 activity, without affecting insulin-stimulation of Ser473 phosphorylation. These results strongly suggest that insulin-stimulated phosphorylation on Ser473 is not dependent on the activity of PDK1 or PKB. Our data is consistent with a model in which phosphorylation on Thr308 and Ser473 occurs via two distinct upstream kinases which are constitutively active at the plasma membrane, and of these, only the Ser473 kinase is staurosporine resistant.

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Figure Legends

Fig. 1. Effect of staurosporine and its derivatives on TPA-stimulated activation of C1-PKBα-ΔPH.

HEK293 cells transiently transfected with HA-C1-PKBα-ΔPH were treated with the indicated concentration of staurosporine (Alexis), CGP 39360, CGP 41251, CGP 42700 (B), CGP 25956, CGP 45910 or CGP 57148B/STI571 (C) for 30 min prior to stimulation with TPA (200 ng/ml, Life Technologies) for 15 min. HA-C1-PKBα-ΔPH was immunoprecipitated and assayed for kinase activity, or analyzed by immunoblotting with phospho-specific antibodies.

Fig. 2. Differential effects of staurosporine on PKB phosphorylation induced by insulin, co-expression of PDK1 or membrane targeting.

HEK293 cells were transiently transfected with wild type HA-PKBα (A, C, D), myristoylated/palmitylated HA-PKBα (D), and myc-PDK1 (C). Cells were treated with the indicated concentration of staurosporine for 30 min prior to stimulation with 0.1 μM insulin (Life Technologies) for 15 min. HA-PKBα was immunoprecipitated and assayed for kinase activity, or analyzed by immunoblotting with phospho-specific antibodies. The phosphorylation status of endogenous PKB was determined by immunoblotting cell lysates (20 μg) with phospho-specific antibodies (B).

Fig. 3. Effect of staurosporine on insulin-stimulated phosphorylation and activation of PKBα mutants.

HEK293 cells transiently transfected with wild type or mutant HA-PKBα were

treated with 0.1 or 1 μM of staurosporine for 30 min prior to stimulation with 0.1 μM insulin for 15 min. HA-PKBα expression was determined by blotting with an anti-HA antibody. PKB activity and phosphorylation was analyzed as for Fig. 1.

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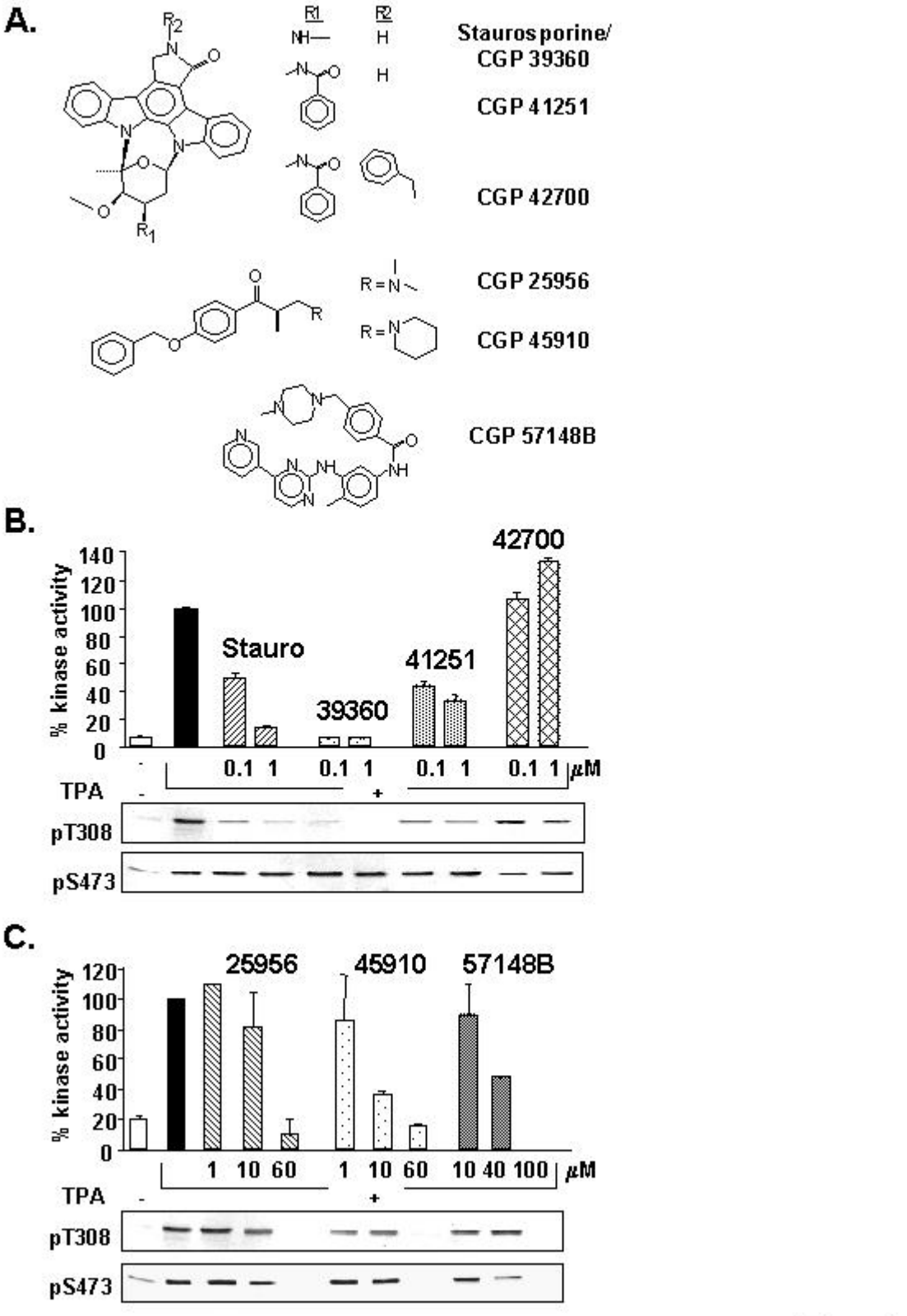
A.
R1
R2
Staurosporine/
CGP 39360
CGP 41251
CGP 42700
CGP 25956
CGP 45910
CGP 57148B
B.
% kinase activity
Stauro
39360
41251
42700
0.1 1 0.1 1 0.1 1 0.1 1 μM
TPA
pT308
pS473
C.
% kinase activity
25956
45910
57148B
1 10 60 1 10 60 10 40 100 μM
TPA
pT308
pS473

Fig. 1

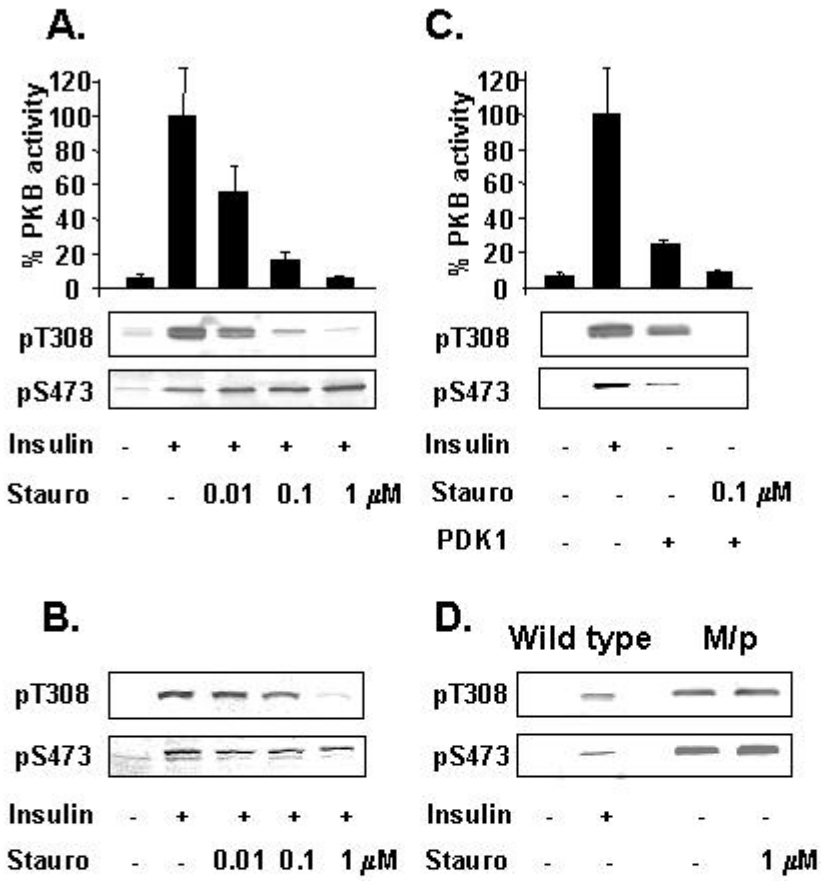
A.
C.
% PKB activity
120
100
80
60
40
20
0
pT308
pS473
Insulin - + + + +
Stauro - - 0.01 0.1 1 μM
Insulin - + - -
Stauro - - - 0.1 μM
PDK1 - - + +
B.
D.
Wild type
M/p
pT308
pS473
Insulin - + + + +
Stauro - - 0.01 0.1 1 μM
Insulin - + - -
Stauro - - - 1 μM

Fig. 2

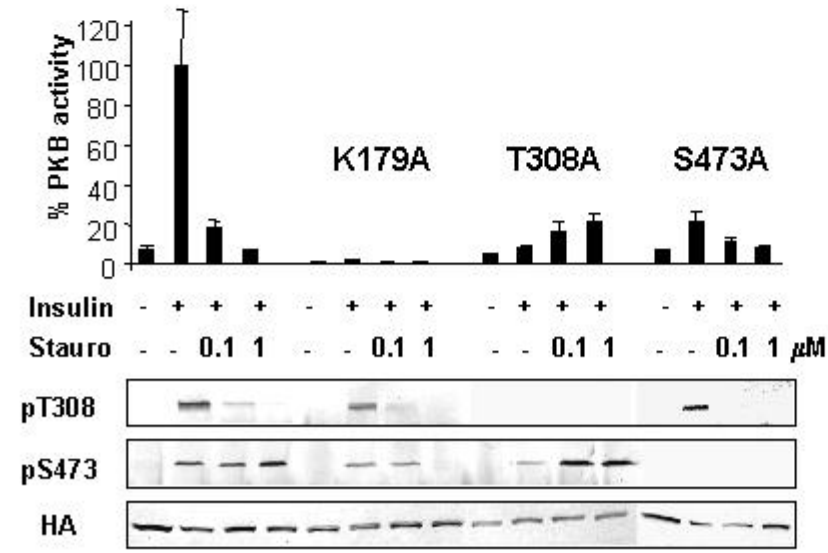
% PKB activity
120
100
80
60
40
20
0
K179A
T308A
S473A
Insulin - + + + - + + + - + + + - + + +
Stauro - - 0.1 1 - - 0.1 1 - - 0.1 1 - - 0.1 1 μM
pT308
pS473
HA

Fig. 3